For Research Use Only

March 3, 2014 Ver. 4

[KLH (TDAR) Rat IgM ELISA KIT]

(Code No.: AKRKM-010)

Please, read this instruction carefully before use.

This kit is manufactured by Shibayagi Co., Ltd.
Use only the current version of Instruction Manual enclosed with the kit! For the detailed assay procedure, refer to Key points for ELISA by movie on our website:
http://www.shibayagi.co.jp/index-E.htm

1. Intended use

KLH (TDAR) Rat IgM ELISA Kit is a sandwich ELISA system for measurement of IgM-type anti-KLH (Keyhole limpet hemocyanin) antibody after inoculation of KLH to rat with high sensitivity. This is helpful in examining TDAR (T-cell dependent antibody reaction) in combination with KLH (TDAR) RAT IgG ELISA KIT. This is intended for research use only.

2. Storage and expiration

When the intact kit is stored at 2-8°C, the kit is stable until the expiration date shown on the label on the box. Reagents, once opened, should be used as soon as possible to avoid losing its optimal assay performance by storage environment.

3. Introduction

ICH (International Conference on Harmonisation of Technical Requirements of Resistration of Pharmaceuticals for Human Use) issues a guideline "Immunotoxicity Studies for Human Pharmaceuticals S8" in 2005. In the guideline, TDAR (T cell Dependent Antibody Reaction) is recommended in case target of a pharmaceutical's immunotoxicity is not identified. It says "The TDAR should be performed using a recognized T-cell dependent antigen (e.g., sheep red blood cells (SRBC) or keyhole limpet hemocyanin (KLH) that results in a robust antibody response". In this study, the production of IgM-type antibody caused by the primary response to e.g., KLH, and IgG-type antibody production by "class-switch" following the secondary response are tested. This kit makes it possible to measure IgM-type anti-KLH antibody in rat blood samples, and most suitable for TDAR test when used in combination with KLH(TDAR) Rat IgG ELISA kit.

4. Assay principle

In Shibayagi's KLH (TDAR) Rat IgM ELISA Kit, standards or samples are incubated in KLH coated wells to capture anti-KLH antibody. After 1 hour incubation and washing, HRP (horse radish peroxidase)-labeled anti-rat IgM antibody is added and incubated for 1 hour together with captured anti-KLH-IgM. After washing, HRP-complex remaining in wells is reacted with a chromogenic substrate (TMB) for 20 minutes, and reaction is stopped by addition of acidic solution, and absorbance of yellow product is measured spectrophotometrically at 450 nm. The absorbance is nearly proportional to anti-KLH-IgM concentration. The standard curve is prepared by plotting absorbance against the standard concentrations. Anti-KLH-IgM concentrations in unknown samples are determined using this standard curve.

5. Precautions

- For professional use only, beginners are advised to use this kit under the guidance of experienced person.
- Wear gloves and laboratory coats when handling assay materials.
- Do not drink, eat or smoke in the areas where assays are carried out.
- In treating assay samples of animal origin, be careful for possible biohazards.
- Ware gloves and goggle and clothing-protection when handling the reaction stopper solution (1M sulfuric acid) and the chromogenic substrate solution (hydrogen peroxide and tetramethylbenzidine). Be careful not to allow the reagent solutions of the kit to touch the skin, eyes and mucous membrane. The reaction stopper and chromogenic substrate solution may cause skin/eyes irritation. In case of contact with these, wash the place thoroughly with

enough water and seek medical attention if necessary.

- The materials must not be pipetted by mouth.
- Residual samples and used tips should be rinsed in 1% formalin, 2% glutal aldehyde, or more than 0.1% sodium hypochlorite solution for more than 1 hour, or be treated by an autoclave before disposal.
- Dispose consumable materials and unused contents in accordance with applicable regional/national regulatory requirements.
- Use clean laboratory glassware.
- In order to avoid dryness of wells, contamination of foreign substances and evaporation of dispensed reagents, never forget to cover the well plate with a plate seal supplied, during incubation.
- ELISA can be easily affected by your laboratory environment. Room temperature should be at 20-25°C strictly. Avoid airstream velocity over 0.4 m/sec. ① (including wind from air conditioner), and humidity less than 30%. ①For airstream, refer to [Assay circumstance] on our web site.

6. Reagents supplied

Components	State	Amount
(A) KLH-coated plate	Use after washing	96 wells/1 plate
(B) Standard anti-KLH rat IgM solution (1000ng/ml) (derived from rat)	Concentrated. Use after dilution	200 µl/1 vial
(C) Buffer solution	Ready for use.	100 ml/1 bottle
(D) HRP-conjugated anti-rat IgM antibody	Concentrated. Use after dilution.	100 µl/1 vial
(F) Chromogenic substrate reagent (TMB)	Ready for use.	12 ml/1 bottle
(H) Reaction stopper (1M H_2SO_4) Be careful!	Ready for use.	12 ml/1 bottle
(I) Concentrated washing buffer (10x)	Concentrated. Use after dilution.	100 ml/1 bottle
Plate seal	−	3 sheets
Instruction Manual	−	1 copy

7. Equipments required but not supplied ☐Use as a check box

☐Purified water (distilled water)
☐Test tubes for preparation of standard solution series.
☐Glassware for dilution of washing buffer (a graduated cylinder, a bottle)
☐Pipettes (disposable tip type). One should be able to deliver 10-20 µl precisely, and another for 50-500 µl.
☐Syringe-type repeating dispenser like Eppendorf multipette plus which can dispense 50 µl.
☐Paper towel to remove washing buffer remaining in wells.
☐A vortex-type mixer.
☐A shaker for 96 well-plate (600-1200rpm)
☐An automatic washer for 96 well-plate (if available), or a wash bottle with a jet nozzle. (refer to our web movie [Washing of microplate])
☐A 96 well-plate reader (450nm ±10nm, 620nm: 600-650nm)
☐Software for data analysis, if available. Shibayagi is proposing the use of assay results calculation template for EXCEL. Please check our website (http://www.shibayagi.co.jp/en/tech_003.html).

8. Preparation of reagents

◆Bring all reagents of the kit to room temperature (20-25 °C) before use.

◆Prepare reagent solutions in appropriate volume for your assay. Do not store the diluted reagents.

【Concentrated reagents】

[(B) Standard anti-KLH rat IgM solution (1000ng/ml)]

Make a serial dilution of original standard solution to prepare each standard solution. Example is shown below.

Volume of standard solution	Buffer solution	Concentration (ng/ml)
Original solution : 100 µl	400 µl	200
200 ng/ml solution : 200 µl	200 µl	100
100 ng/ml solution : 200 µl	200 µl	50
50 ng/ml solution : 200 µl	200 µl	25
25 ng/ml solution : 200 µl	200 µl	12.5
12.5 ng/ml solution : 200 µl	200 µl	6.25
6.25 ng/ml solution : 200 µl	200 µl	3.13
0 (Blank)	200 µl	0

[(D) HRP-conjugated anti-rat IgM antibody]

Prepare working solution by dilution of (D) with the buffer solution (C) to 1:100.

[(I) Concentrated washing buffer (10x)]

Dilute 1 volume of the concentrated washing buffer (10x) to 10 volumes with deionized water to prepare working solution. Example: 100 ml of concentrated washing buffer (10x) and 900ml of deionized water.

【Storage and stability】

[(A) KLH-coated plate]

If seal is not removed, put the strip back in a plastic bag with zip-seal originally used for well-plate container and store at 2-8 °C. The strip will be stable until expiration date.

[(B) Standard anti-KLH rat IgM solution (1000ng/ml)]

Standard solutions prepared above should be used as soon as possible, and should not be stored.

[(C) Buffer solution] and [(F) Chromogenic substrate reagent]

If not opened, store at 2-8 °C. It maintains stability until expiration date. Once opened, we recommend using them as soon as possible to avoid influence by environmental condition.

[(D) HRP-conjugated anti-rat IgM antibody]

Unused working solution (already diluted) should be disposed. The rest of the undiluted solution will be stable until expiration date if stored tightly closed at 2-8 °C.

[(H) Reaction stopper (1 M H_2SO_4)]

Close the stopper tightly and store at 2-8 °C. It maintains stability until expiration date.

[(I) Concentrated washing buffer (10x)]

The rest of undiluted buffer: if stored tightly closed at 2-8 °C, it is stable until expiration date. Dispose any unused diluted buffer.

9. Technical tips

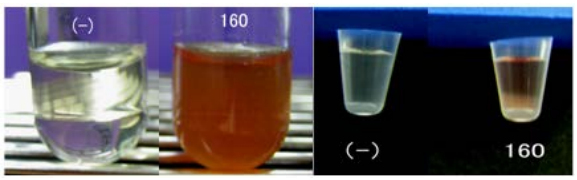
- In manual operation, proficiency in pipetting technique is recommended.
- The reagents are prepared to give accurate results only when used in combination within the same box. Therefore, do not combine the reagents from kits with different lot numbers. Even if the lot number is the same, it is best not to mix the reagents with those that have been preserved for some period.
- Be careful to avoid any contamination of assay samples and reagents. We recommend the use of disposal pipette tips, and 1 tip for 1 well.
- Optimally, the reagent solutions of the kit should be used immediately after reconstitution. Otherwise, store them in a dark place at 2-8 °C.
- Time the reaction from the pipetting of the reagent to the first well.
- Prepare a standard curve for each assay.
- Dilution of the assay sample must be carried out using the buffer solution provided in the kit.
- The chromogenic substrate (TMB) solution should be almost colorless before use. It turns blue during reaction, and gives yellowish color after addition of reaction stopper. Greenish color means incomplete mixing.
- To avoid denaturation of the coated KLH, do not let the plate go dry.
- As the KLH-coated plate is module type of 8wells x 12 strips, each strip can be separated by cutting the cover sheet with a knife and used independently.
- When ELISA has to be done under the airstream velocity over 0.4 m/sec. and the humidity less than 30%, seal the well plate with a plate seal and place the well plate in an incubator or a styrofoam box in each step of incubation. For more details, watch our web movie [Assay circumstance].

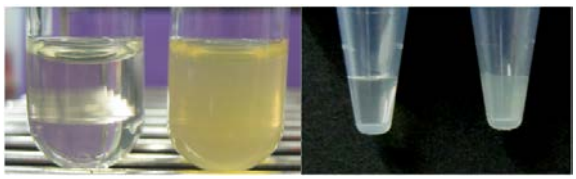
10. Preparation of samples

This kit is intended to measure IgM-type anti-KLH (Keyhole limpet hemocyanin) antibody in rat serum or plasma. The necessary sample volume for the standard procedure is 10 µl. Samples should be immediately assayed or stored below –35 °C for several days. Defrosted samples should be mixed thoroughly for best results. Don't repeat freeze and thaw.

Hemolytic and hyperlipemic samples are not suitable.

* To avoid influence of blood (high lipid or hemolysis, etc.), if your original samples have heavy chyle or hemolysis as the pictures below, do not use them for assay. Abnormal value might be obtained with hemolysis above 160mg/dL with this kit.





Normal	Hemolysis 160mg/dL	normal	Hemolysis 160mg/dL	Normal	Chyle Highly lipid sample	Normal	Chyle Highly lipid sample

If presence of interfering substance is suspected, examine by dilution test at more than 2 points. Turbid samples or those containing insoluble materials should be centrifuged before testing to remove any particulate matter.

Make sure to dilute samples more than 200x to avoid any nonspecific reaction. Recommended dilution rate is 200-20,000x depending on the antibody titer. Dilution should be carried out with the buffer solution of the kit using small test tubes before assay so as to be within the standard curve range. Dilution rate should be different depending on the immune or sampling conditions.

Example of dilution: Rate	(20x)		200x		2,000x		20,000x
Sample (µl)	10	⌜	20*	⌜	20*	⌜	20*
Buffer (µl)	190	⌞	180	⌞	180	⌞	180

*One rank lower diluted sample

Storage and stability

Sample is stable at 2-8°C within a week. If you have to store assay samples for a longer period, snap-freeze samples and keep them below –35°C. Avoid repeated freezing and thawing cycles.

11. Assay procedure

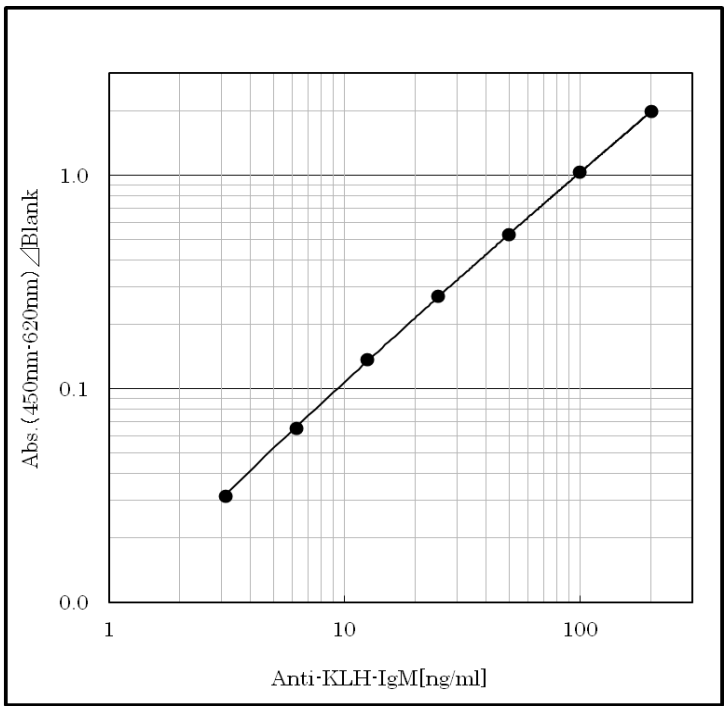
Remove the cover sheet of the 96 well-plate after bringing up to room temperature.

(1) Wash the KLH-coated plate (A) by filling the well with washing buffer and discard 3 times(*②), then strike the plate upside-down onto several layers of paper towels to remove residual buffer in the wells.
(2) Pipette 50 µl of standards or diluted samples to the wells designated for each.
(3) Shake the plate gently on a plate shaker (*③).
(4) Stick a plate seal (*④) on the plate and incubate for 1 hour at 20-25°C.
(5) Discard the reaction mixture and rinse wells as step (1).
(6) Pipette 50 µl of HRP-conjugated anti-rat IgM antibody (D) to all wells, and shake as step (3).
(7) Stick a plate seal (*④) on the plate and incubate the plate for 1 hour at 20-25°C.
(8) Discard the reaction mixture. Rinse wells as step (1).
(9) Pipette 50 µl of chromogenic substrate reagent (F) to wells, and shake as step (3).
(10) Stick a plate seal (*④) on the plate and incubate the plate for 20 minutes at 20-25°C.
(11) Add 50 µl of the reaction stopper (H) to all wells and shake as step (3).
(12) Measure the absorbance of each well at 450 nm (reference wavelength, 620*nm) using a plate reader within 30 minutes.

*Refer to the page 7 for notes of *②, *③ and *④.

12. Calculations

(1) Prepare a standard curve using a two-way logarithmic section paper by plotting absorbance* (Y-axis) against KLH IgM concentration (ng/ml) on X-axis. Physiological or pathological situation of animals should be judged comprehensively taking other examination results into consideration.
(2) Using the standard curve, read the KLH IgM concentration of a sample at its absorbance*, and multiply the assay value by dilution factor. Though the assay range is wide enough, in case the absorbance of some samples is higher than that of the highest standard, please repeat the assay after proper dilution of samples with the buffer solution. * We recommend the use of 3rd order regression curve for log-log plot, or 4 parameters method for log-normal plot in computer calculation.



KLH rat IgM assay standard curve (an example)
Absorbance may change due to assay situation.

13. Performance characteristics

- Assay range

The assay range of the kit is 3.13 ~ 200 ng/ml.

- Specificity

The HRP-conjugated anti-rat IgM antibody of this kit is specific to anti-rat IgM.
The cross-reactivity with anti-rat IgG is less than the detection limit.

- Precision of assay

Within assay variation (2 samples, 5 replicates assay), the mean CV was less than 5%.

- Reproducibility

Between assay variation (3 samples, 4 days, 4 replicates assay), the mean CV was less than 5%

- Recovery test

Standard anti-KLH rat IgM was added in 3 concentrations to 2 serum samples and were assayed. The recoveries were 94.8 ~102%

- Dilution test

2 serum samples were serially diluted by 3 steps.
The dilution curves showed linearity with $R^2 = 0.998 \sim 0.999$.

14. Trouble shooting

- Low absorbance in all wells

  Possible explanations:

  1) The standard or samples might not be added.
  2) Reagents necessary for coloration such as HRP-conjugated anti-rat IgM antibody or TMB might not be added.
  3) Wrong reagents related to coloration might have been added. Wrong dilution of HRP-conjugated anti-rat IgM antibody.
  4) Contamination of enzyme inhibitor(s).
  5) Influence of the temperature under which the kits had been stored.
  6) Excessive hard washing of the well plate.
  7) Addition of TMB solution soon after taking out from a refrigerator might cause poor coloration owing to low temperature.

- Blank OD was higher than that of the lowest standard concentration (3.13 ng/ml).

  Possible explanations:
  Improper or inadequate washing. (Change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP-conjugated anti-rat IgM antibody.)

- High coefficient of variation (CV)

  Possible explanation:

  1) Improper or inadequate washing.
  2) Improper mixing of standard or samples.
  3) Pipetting at irregular intervals.

- Q-1: Can I divide the plate to use it for the other testing?

  A-1: Yes, cut off the clear seal on the plate with cutter along strip. Put the residual plate, which is still the seal on, in a refrigerator soon

- Q-2: I found there contains liquid in 96 well-plate when I opened the box. What is it?

  A-2: When we manufacture 96 well-plate, we insert preservation stabilizer in wells.

For detailed FAQS and explanations, refer to **"Trouble shooting and Important Points in Shibayagi's ELISA kits"** on our website (http://www.shibayagi.co.jp/en/tech_004.html).

Summary of assay procedure ☐ : Use as a check box

*First, read this instruction manual carefully and start your assay after confirmation of details.

For more details, watch our web movie [ELISA by MOVIE] on our website.

☐Bring the well-plate and all reagents to 20-25°C for 2 hours.

☐Concentrated washing buffer must be diluted to 10 times by purified water.

☐Standard solution dilution example:

Concentration (ng/ml)		200	100	50	25	12.5	6.25	3.13	0
Std. solution (µl)	orig.sol. 100		200*	200*	200*	200*	200*	200*	0
Buffer solution (µl)		400	200	200	200	200	200	200	200

*One rank higher standard.

			Precautions & related info
☐	KLH-coated plate		
☐	↓ Washing 3 times(*②)		*⑥
☐	Diluted Samples / Standards	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C (Standing***)		*⑧ [Assay circumstance]
☐	Meanwhile, Dilute HRP-conjugated anti-rat IgM antibody (D) to 100x with Buffer (C) returned to 20-25°C.		
☐	↓ Washing 3 times(*②)		*⑥
☐	HRP-conjugated anti-rat IgM antibody	50 µl	*⑦ [Handling of pipetting]
☐	↓ Shaking(*③), Incubation for 1 hour at 20-25°C (Standing(*④))		*⑧ [Assay circumstance]
☐	↓ Washing 3 times(*②)		*⑥
☐	Chromogenic substrate (TMB)	50 µl	After dispense, the color turns to blue depending on the concentration.
☐	↓ Shaking(*③), Incubation for 20 min at 20-25°C (Standing(*④))		*⑧ [Assay circumstance]
☐	Reaction stopper (1M H_2SO_4)	50 µl	After dispense, the color turns to yellow depending on the concentration.
☐	↓ Shaking(*③)		Immediately shake.
☐	Measurement of absorbance (450nm, Ref 620nm(*⑤))		Ref. wave cancels the dirt in the back of plate.

*②After dispensing wash buffer to wells, lightly shake the plate on your palm for 10 sec and remove the buffer. Guideline of washing volume: 300µl/well for an automatic washer and for a pipette if the washing buffer is added by pipette. In case of washing by using 8 channel pipette, sometimes the back ground tends to be high. If so, change washing frequency from 3 times to 4-6 times at the constant stroke after the reaction with HRP conjugated streptavidin.
Standard of plate-washing pressure: 5-25ml/min. (Adjust it depending on the nozzle's diameter.) Refer to our web movie [Washing of microplate].

*③Guideline of shaking: 600-1,200rpm for 10 seconds x 3 times.

*④Seal the plate during the reaction after shaking. Peel off the protective paper from the seal and stick the seal on the plate. Do not reuse the plate seal used once.

*⑤600-650 nm can be used as reference wavelength.

*⑥After removal of wash buffer, immediately dispense the next reagent.

*⑦Refer to our web movie [Handling of pipetting].

*⑧Refer to our web movie [Assay circumstance]

Worksheet example

	Strip 1&2	Strip 3&4	Strip 5&6	Strip 7&8	Strip 9&10	Strip 11&12
A	200 ng/ml	Sample 1	Sample 9	Sample 17	Sample 25	Sample 33
B	100 ng/ml	Sample 2	Sample 10	Sample 18	Sample 26	Sample 34
C	50 ng/ml	Sample 3	Sample 11	Sample 19	Sample 27	Sample 35
D	25 ng/ml	Sample 4	Sample 12	Sample 20	Sample 28	Sample 36
E	12.5 ng/ml	Sample 5	Sample 13	Sample 21	Sample 29	Sample 37
F	6.25 ng/ml	Sample 6	Sample 14	Sample 22	Sample 30	Sample 38
G	3.13 ng/ml	Sample 7	Sample 15	Sample 23	Sample 31	Sample 39
H	0	Sample 8	Sample 16	Sample 24	Sample 32	Sample 40

Assay worksheet

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H												

[Storage condition] Store the kit at 2-8°C (Do not freeze).
[Term of validity] 6 months from production (Expiration date is indicated on the container.)

This kit is manufactured by **Shibayagi Co., Ltd.**
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